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14. ABSTRACT
Work was initiated to test two polyethylene glycolated (PEGylated) forms of bovine serum amine oxidase (SAO) as effective treatments for breast cancer using a mouse model. Hopefully, this approach, or a variation thereof, could be used as a new a therapy for breast and other human cancers. Currently, a large quantity of pure bovine SAO is in hand, which was obtained from ~ 10 gallons of fresh cow blood. A final purification step was used to produce large quantities of extremely pure SAO. Attempts to find a cost-effective, practical method for the degycosylation of SAO were unsuccessful. Using different PEGylating reagents, we attempted to obtain several PEG derivatives of SAO. Although preliminary test indicated that SAO could be PEGylated at a fairly high level, efforts to repeat this on a large scale failed. Hence, it has not been possible to test any PEG-SAO derivatives in non-tumorigenic mice, or mice harboring human breast-cancer tumors. Effort to find efficient ways to PEGylate SAO at a high level will continue. High level PEGylation occurred with freshly prepared SAO. Although about 3 grams of highly pure enzyme, it is proposed that freshly prepared oxidase is required. In the future, will carry out smaller scale purifications, and only use the freshly prepared SAO for PEGylation, & we will try different PEGylation reagents. Hopefully, the problem can be remedied.

15. SUBJECT TERMS
cancer, tumor, breast cancer treatment, polyamines, serum amine oxidase, plasma amine oxidase, drug design

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INTRODUCTION

The goal of this research was to obtain large quantities of two polyethylene glycolated (PEGylated) forms of bovine serum anime oxidase (SAO) that would be tested as anti-tumor agents in mouse breast cancer models. After injecting PEG-encapsulated SAO into the bloodstream, it would target specifically tumors due to the abnormally large pore in the blood vessels engulfing tumors. PEGylated proteins are known to have a long half-life in the blood and are non-antigenic. Hence, over time, they would accumulate in tumors but not normal tissues (1-3). To prevent the SAO from interfering or substituting for the endogenous murine SAO (i.e., in its role as Vascular Adhesion Protein-1, VAP-1; ref. 4) in the blood, we sought to degylcosylate the bovine SAO before PEGylating. VAP-1 is important for leukocyte migration from the blood into the tissues.

SAO oxidizes efficiently N^1-acetyl-spermine and N^1-acetyl-spermidine. These acetylated polyamines are exported from tumor cells at high levels. Presumably, toxic products will be generated locally in sufficient quantities, via the oxidation of the acetylated polyamines by modified SAO, to slow or arrest the growth, or kill tumor cells without harming substantially non-cancerous tissues (1, 2). A recent report indicates that administering PEG-proteins to animals or human poses no significant risk (5).

BODY

***TASK 1*. Prepare sufficient quantities of two PEG-derivatives of bovine serum amine oxidase (SAO).**

A. **Purification of bovine serum amine oxidase (SAO**). The enzyme was purified using established procedures (6-8). It required nearly two month, full-time for the PI to complete the purification:

Step 1: Processing of bovine blood. Forty Liters (10.6 gal) fresh bovine blood obtained from a local slaughterhouse was immediately treated with $1/10^{th}$ volume of sodium citrate, and was put on ice for transport to the lab. The serum was centrifuged at 10,000 rpm in 380 mL centrifuge bottles for 30 min to remove cells.

Step 2: $(NH_4)_2SO_4$ treatment. The resulting red liquid was made 35 % $(NH_4)_2SO_4$ by slowly adding solid salt to the stirring solution, over 1 h period. Once all the $(NH_4)_2SO_4$ dissolved, the solution was stirred for another hour, and then centrifuged at 10,000 rpm in 380 mL centrifuge bottles for 30 min. The supernatant was collected, and brought to 55 % $(NH_4)_2SO_4$ as before. After centrifugation, the resulting pellet, which had the majority of the SAO activity, was redissolved in ~ 1.5 L of 10 mM KP_i, pH 7.0. The liquid was centrifuge to remove insolubles and then concentrated in 350 mL Amicon pressure concentrators using YM30 membranes. Once the volume of the sample reached ~ 50 mL, it was replenished with ~ 300 mL of the buffer. This process was repeated four times. The final volume was brought to 3 L with the 10 mM buffer.

Step 3. DEAE-cellulose ion-exchange chromatography. The sample was applied to a 14 x 35 cm column packed with Watman DEAE-cellulose. The flow rate was about 60 mL/min. The column was washed with 4 L of 10 mM KP_i, pH 7.0 buffer, and the protein was eluted with a 10 L gradient from 0 to 150 mM KCl in the same buffer, followed by 3 L of 10 mM KP_i, pH 7.0 containing 150 mM KCl. Thirty six-mL fractions were collected, and all fraction that had all but a trace of SAO activity were pooled. Assays were done using the procedure described in reference 9 using 5 mM benzylamine as the

substrate. One to ten microliters of each fraction were added to 50 μL of the assay mixture. The development of the red chromophore color was following to determine the relative activities. The pooled-fractions solution was concentrated using two 400-mL Amicon pressure concentrator with YM30 membranes. After concentrating to ~100 mL, 250 mL of 100 mM KP_i, pH 7.2 was added and the liquid again concentrated to ~ 50 mL. This step was repeated twice more. The final concentration was brought to 100 mL.

Step 4. **Concanavalin-A Sepharose 4B affinity chromatography**. Fifty milliliters of the solution was applied to a 2.5 x 55 cm column packed with Con-A Sepharose 4B (Sigma). The sample was washed with 100 mM KP_i, pH 7.2 buffer until the A_{280} readings of the elutant fell to < 0.05. The column was then eluted with a 250 mM methyl α-mannopyranoside in 100 mM KP_i, pH 7.2 solution. All fractions containing activity were pooled. The 2nd 50 mL portion of the protein sample was processed in the same manner. The pooled fractions were concentrated and washed as above with 250 mM KP_i, pH 7.0 buffer. The liquid was brought to 100 mL with this buffer.

Step 5. **ULTROGEL AcA 34 molecular sieving chromatography**. Half of the sample was applied to a 5 x 95 cm column packed with ULTROGEL AcA 34 resin (BIOSEPRA) equilibrated with 250 mM KP_i, pH 7.0 buffer. The protein was eluted with this buffer at a flow rate of about 30 mL/hour. All fractions containing but a trace of activity were pooled. The 2nd half of the sample was chromatographed in the same manner. At this point, the SAO was fairly pure and it had the expected specific activity (6). The pooled fractions were concentrated, and washed into 100 mM KP_i, pH 7.2 buffer containing 100 mM KCl. The concentration was about 30 mg/mL. The enzyme was stored in 10 mL and 1.5 mL fractions at -80 °C. The final yield of SAO was about 3 grams. The specific activity (k_{cat}) was 0.24 μmol/(min mg) (K_M = 1.7 mM) (25 mM KP_i, I = 0.075, pH 7.0, air-saturated buffer, T = 30 ° C) using the typical substrate benzylamine. However, we found spermine to be a much better substrate in the same buffer (air saturated, T = 30 ° C); k_{cat} = 6.6 μmol/(min mg) & K_M = 10.1 μM. For these assay we used the ABTS/Peroxidase assay describe in the literature (10); ABTS is 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid).

Step 6. **Macro-Prep Ceramic Hydroxyapatite, Type I chromatography**. A column (1.6 x 10 cm; GE Healthcare HR 16/10) was dry packed with Macro-Prep Ceramic Hydroxyapatite, Type I chromatography material (Bio-Rad). The column was washed extensively with degassed 1 M KP_i, pH 7.2 buffer to remove absorbed material and fines, and to eliminate all air voids. The column was then washed with 1 mM KP_i, pH 7.2 buffer. Just before using, the enzyme solution was exchanged by several concentration/5 mM KP_i, pH 7.2 dilution cycles washed using Centricon (Amicon) YM30 centrifuge concentrators. Six hundred microliter of the SAO solution (12.5 mg/mL) was diluted with 1.35 mL water just prior to injecting the entire volume onto the column: flow rate – 3 mL/min; wash 6 min with water, then a linear gradient from 0 to 0.5 M KP_i, pH 7.25 buffer in 30 min (Fig. 1yy). PAGE indicated that the SAO was pure, with only a low trace of other proteins.

B . Deglycosylation of bovine SAO. The enzyme has, at least, 3 glycosylated sites (11). The carbohydrate moieties are required for its role as VAP-1 (4). Hence, to minimize side-effects that might occur when inject into teat animals, we attempted to remove the attached carbohydrate to the greatest extent feasible. To accomplish this we used the ProZyme Glyko enzymatic degylcosylation kit. Initially, we tried to deglycosylate 0.1 mg of SAO according to the manufactures instructions. Even after prolonged incubation (2 weeks; recommended 1-5 days), very little degylcosylation occurred as judged by SDS-PAGE (12). Even at 5-time higher deglycosylating enzymes, little deglycosylation occurred after 5 days. It was estimated that it would cost many thousands of dollars to deglycosylate a few milligrams of SAO, and still be too little for our studies. Hence, this endeavor was terminated. This required about 2 months part-time work by the PI.

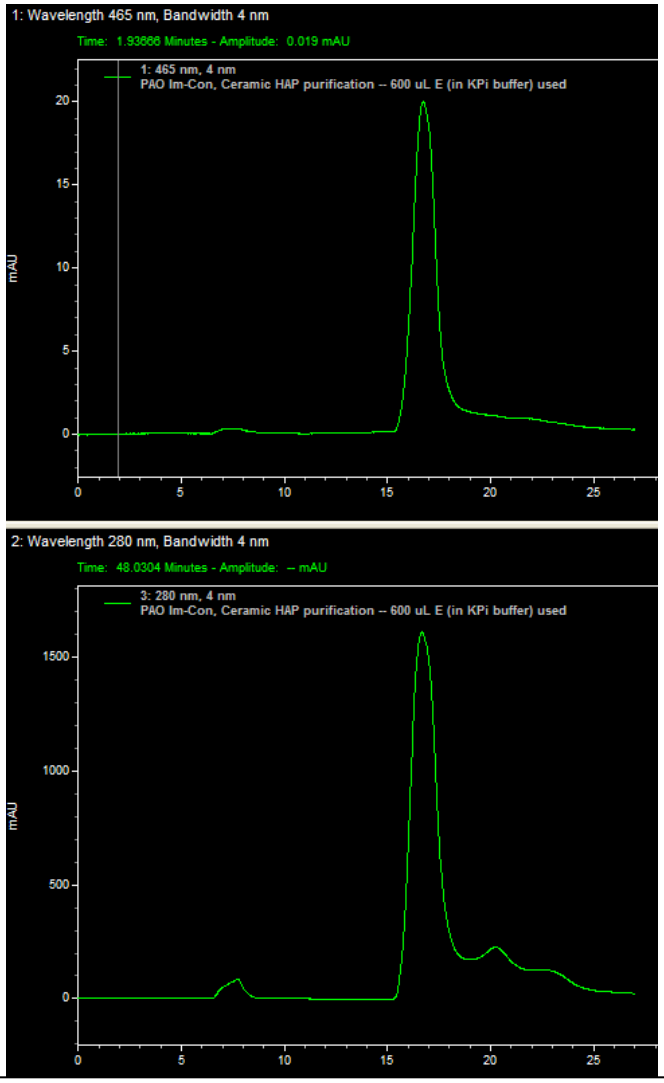

Figure 1yy. Final step of bovine SAO purification - Macro-Prep Ceramic Hydroxyapatite, Type I chromatography. The flow rate was 3 mL/min. After injecting the sample, the column was washed 6 min with water, and the protein eluted with a linear gradient from 0 to 0.5 M KP_i, pH 7.25 buffer in 30 min. The upper chromatogram is a plot of A_{465} vs time, and the lower one is a plot of A_{280} vs time. A_{465} is the approximat maximum absorbance of SAO in the visible region. A SAO-containing fraction, from ~ 15.5 min to ~ 18.2 min, was collected.

C. PEGylation of SAO. Two miligrams of SAO in 1 mL of 50 mM KPi, pH 7.4 at 25 °C was reacted with with either 4.7 mg Sunbright ME-050CS (5 kDa PEG) for 1 h, 19 mg Sunbright ME-200CS (20 kDa PEG) for 1 h, or both (1 h, first with 4.7 mg ME-050CM, and then 1 h with ME-200CM). These *N*-hydroxysuccinimide ester derivatives of mPEG (methoxy-PEG) were purchased from NOF, American Corporation, Tokyo, White Plains, NY. They react specifically with the ε-amino group of Lys residues. From the structure of SAO, we estimated that there are ~ 13 surface Lys residues/subunit of the homo-dimeric oxidase; the level of PEGylated reagent was added at about a 3.5 molar excess to surface Lys.

We use the TNBS (trintirobenzene sulfonate) method described in the literature to measure free amino groups of a protein (from Lys and the N-terminal amino acid) of SAO (13). With this method, we found 15.3 free amino groups/subunit for unadultered SAO, 10.5 free amino groups/subunit for SAO-5 kDa PEG, 11.5 free amino groups/subunit for SAO-20 kDa PEG, and 9.6 free amino groups/subunit for SAO-5/20 kDa PEG. This gives about five 5-kDa PEG groups, about four 20-kDa PEG groups, and about six 5/20-kDa groups per subunit, respectively. We found that SAO treated with both PEGylating reagents lost a significant amount of activity, so it was not studied further. Below are the steady-state kinetic parameters for various forms of SAO. The k_{cat} values decrease slightly, and the K_M values changed modestly for the PEGylated derivatives.

SAO: k_{cat} = 6.6 μmol/(min mg), K_M = 10.1 μM

SAO-5 kDa PEG: k_{cat} = 6 μmol/(min mg), K_M = 10.7 μM (~5 PEG/subunit or 10 PEG/SAO)
SAO-20 kDa PEG: k_{cat} = 5.6 μmol/(min mg), K_M = 8.6 μM (~4 PEG/subunit or 8 PEG/SAO)

We were encouraged by the result because we got significant PEGylation that did not affect the activity very much.

Several months later, we repeated this work, only to find that we got < 1 PEG group/subunit with either reagent. Another try gave the same results. Hence, we switch to similar PEGylating reagents that also reacts with Lys ε-amino groups; mPEG-SPA, 5 kDA and mPEG-SPA, m20 kDa (Nektar Therapeutics, San Carlos, CA; SPA indicates succinimidyl proprionate). Earlier, we used these to PEGylate human peroxisomal polyamine oxidase at a high level, with little effect on the kinetic parameters (unpublished work). However, for SAO, again we got < 1 Lys modified/subunit with both reagents. Finally, we tried mPEG-PNPC (*p*-nitrophenylcarbonate-PEG; M-NPC-5K and N-NPC-20K from Laysan Bio., Inc.). We use essentially the same methods as reported in reference 14 to derivatize the free amino groups of SAO. Once again, we obtained SAO with very little PEGylation. This is surprising because it was reported the ~60 % of the surface Lys residues are modified using this procedure (14). Total time for this aspect was about 3-4 months full- and part-time work by the PI.

Our endeavors to obtain high quantities of usable PEG derivative of SAO will continue. We plan to try other PEGylating reagents, including branched PEGylating compounds.

***TASK 2*. Test the general toxicity of the two PEG-SAO derivatives.**

This work requires injecting several non-tumorigenic mice with one PEG-SAO derivative, and injecting another group of mice with the second derivative. Since we have yet to procure any viable PEG-SAO derivative (*Task 1*), we cannot proceed with this phase of the research.

***TASK 3*. Test each PEG-SAO conjugate as an antitumor agent using mice with implanted human tumors.**

This task could only have been initiated if we had prepared a large amount of the two PEGylated forms or bovine SAO (***TASK 1***), and we have tested these in nontumorigenic mice (***TASK 2***).

KEY RESEARCH ACCOMPLISHMENTS

- Procured a large quantity of pure bovine SAO.
- Developed a procedure to obtain extremely pure SAO for deglycosylation and PEGylation.
- Attempted to efficiently and cost-effectively degycosylate bovine SAO. After 2 months of work, this endeavor was deemed untenable.
- Tried to develop methods to produce two PEGylated derivatives of bovine SAO; one form of SAO would have 5 kDa EG groups attached, and the other form of SAO would have 20 kDa PEG groups attached. Using several PEGylating reagents, we failed to find a procedure that yielded high-level PEGylation of SAO.

REPORTABLE OUTCOMES

The only reportable outcome is that we have obtained the requisite amount of pure bovine SAO for the remainder of our research on this project, and that we are attempting to developed methods to PEGylated the enzyme. There are no publications, abstracts etc. resulting from this research.

CONCLUSION

We conclude that PEGylation works well only with freshly purified SAO. Perhaps, even when stored in frozen solution, this amine oxidase self-oxidizes some or all of the ε-amino groups of surface Lys. However, if occurring, this process does not seem to affect the solubility or activity of SAO. In the future, we will only use freshly prepared SAO, and carry out the purification on a smaller scale.

Since we have not yet done any animal work, we cannot report any conclusions. If our hypothesis is correct, the treatment may one day be an effective anticancer therapy for human patients.

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APPENDICE

NONE

SUPPORTING DATA

NONE

BIBLIOGRAPHY OF PUBLICATIONS & MEETING ABSTRACTS

NONE

SALARIED EMPLOYEES

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